Impact of Phlebotomine Sand Flies on U.S. Military Operations at Tallil Air Base, Iraq: 4. Detection and Identification of *Leishmania* Parasites in Sand Flies

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ABSTRACT Sand flies collected between April 2003 and November 2004 at Tallil Air Base, Iraq, were evaluated for the presence of *Leishmania* parasites using a combination of a real-time *Leishmania*-generic polymerase chain reaction (PCR) assay and sequencing of a 360-bp fragment of the glucose-6-phosphate-isomerase (GPI) gene. A total of 2,505 pools containing 26,574 sand flies were tested using the real-time PCR assay. *Leishmania* DNA was initially detected in 536 pools; however, after extensive retesting with the real-time PCR assay, a total of 456 pools were considered positive and 80 were considered indeterminate. A total of 532 samples were evaluated for *Leishmania* GPI by sequencing, to include 439 PCR-positive samples, 80 PCR-indeterminate samples, and 13 PCR-negative samples. *Leishmania* GPI was detected in 284 samples that were sequenced, to include 281 (64%) of the PCR-positive samples and 3 (4%) of the PCR-indeterminate samples. Of the 284 sequences identified as *Leishmania*, 261 (91.9%) were *L. tarentolae*, 18 (6.3%) were *L. donovani*-complex parasites, 3 (1.1%) were *L. tropica*, and 2 were similar to both *L. major* and *L. tropica.* Minimum field infection rates were 0.09% for *L. donovani*-complex parasites, 0.02% for *L. tropica*, and 0.01% for the *L. major/tropica*-like parasite. Subsequent sequencing of a 600-bp region of the "Hyper" gene of 12 of the *L. donovani*-complex parasites showed that all 12 parasites were *L. infantum*. These data suggest that *L. infantum* was the primary leishmanial threat to U.S. military personnel deployed to Tallil Air Base. The implications of these findings are discussed.

KEY WORDS Sand flies, *Leishmania*, Iraq, detection, polymerase chain reaction

The prevention of infectious diseases that could impact on military operations is a key tenet of military medicine. During contingency operations (e.g., wars, peace-keeping missions, and humanitarian missions), the U.S. military routinely deploys specialized medical units that are capable of assessing the infectious disease threat, and, if warranted, implementing programs to protect our forces from these diseases. Units that are primarily responsible for assessing the infectious disease threat include Army Area Medical Laboratories, Navy Forward-Deployed Preventive Medicine Units, and Air Force Biological Assessment Teams. Once an infectious disease threat has been identified, Army, Navy, and Air Force Preventive Medicine Units are responsible for implementing prevention and control programs.

Historically, leishmaniasis has been a major cause of infectious disease morbidity among military personnel deployed to the Middle East (Kinnamon et al. 1979). During World War II, 1,000–1,500 cases of cutaneous leishmaniasis and 50–75 cases of visceral leishmaniasis occurred in allied forces in the Middle East (Most 1968). During Operation Desert Storm, 20 cases of cutaneous leishmaniasis and 12 cases of viscerotropic leishmaniasis were diagnosed in the 697,000 allied service members deployed to the Arabian Peninsula in 1990 and 1991 (Magill et al. 1993, Martin et al. 1998). Most recently, an outbreak of cutaneous leishmaniasis caused by *Leishmania major* Yakimoff and Schokhor occurred in U.S. military personnel deployed to Iraq during Operation Iraqi Freedom (Centers for Disease

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14. ABSTRACT

Sand ?ies collected between April 2003 and November 2004 at Tallil Air Base, Iraq, were evaluated for the presence of Leishmania parasites using a combination of a real-time Leishmaniageneric polymerase chain reaction (PCR) assay and sequencing of a 360-bp fragment of the glucose- 6-phosphate-isomerase (GPI) gene.Atotal of 2,505 pools containing 26,574 sand ?ies were tested using the real-time PCR assay. LeishmaniaDNAwas initially detected in 536 pools; however, after extensive retesting with the real-time PCR assay, a total of 456 pools were considered positive and 80 were considered indeterminate. A total of 532 samples were evaluated for Leishmania GPI by sequencing to include 439 PCR-positive samples, 80 PCR-indeterminate samples, and 13 PCR-negative samples. Leishmania GPI was detected in 284 samples that were sequenced, to include 281 (64%) of the PCR-positive samples and 3 (4%) of the PCR-indeterminate samples. Of the 284 sequences identi?ed as Leishmania, 261 (91.9%) were L. tarentolae, 18 (6.3%) were L. donovani-complex parasites, 3 (1.1%) were L. tropica, and 2 were similar to both L. major and L. tropica. Minimum ?eld infection rates were 0.09% for L. donovani-complex parasites, 0.02% for L. tropica, and 0.01% for the L. major/tropica-like parasite. Subsequent sequencing of a 600-bp region of the ?Hyper? gene of 12 of the L. donovanicomplex parasites showed that all 12 parasites were L. infantum. These data suggest that L. infantum was the primary leishmanial threat to U.S. military personnel deployed to Tallil Air Base. The implications of these ?ndings are discussed.

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Control and Prevention 2003, 2004; Pehoushek et al. 2004). As of November 2004, 1,178 cases of cutaneous leishmaniasis had been identified in U.S. military personnel (Lay 2004). However, this is probably an underestimate, and the actual number of cases as of January 2009 was estimated to be between 3,000 and 5,000 cases (COL P. Weina, personal communication).

A variety of methods have been used for the detection and identification of *Leishmania* parasites in sand flies. One of the earliest methods was the dissection and visual examination of the digestive tract for the presence of *Leishmania* parasites under a microscope. Although this method is not appropriate for use during military operations because it is time consuming and requires a high level of training, visual observation of *Leishmania* parasites in the vector remains the best method of conclusively incriminating a particular species of sand fly as a vector and is still widely used (Adini et al. 1998, Rossi et al. 2009). The development of immunological methods in general, and the enzyme-linked immunosorbent assay (ELISA) in particular, revolutionized the field of vector diagnostics (Beier 2002). The ELISA assay has been widely used to evaluate sand flies for the presence of *Leishmania* parasites (Adini et al. 1998). Although ELISA assays are capable of testing large numbers of arthropods very rapidly, they are not routinely used during military operations because of the amount of equipment required and reliance on a cold chain. The need for assays that could be readily used for vector surveillance in areas without extensive laboratory facilities and/or a cold chain (e.g., during military deployments) led to the development of a series of hand-held immunochromatographic assays used for malaria (Ryan et al. 2001) and arbovirus (Ryan et al. 2003) surveillance. The Walter Reed Army Institute of Research (WRAIR, Silver Spring, MD) and VecTOR Test Systems (Thousand Oaks, CA) recently initiated efforts to develop a hand-held assay for the detection of *Leishmania* parasites in sand flies.

Although hand-held immunochromatographic assays are ideal for field use, specificity and sensitivity of the assays can be lower than desired. Polymerase chain reaction (PCR) assays are typically highly sensitive and specific and can serve as stand-alone screening assays or as confirmatory assays. PCR assays targeting a wide variety of genes or genomic sequences have been widely used to detect *Leishmania* parasites in sand flies (Paiva et al. 2006, Rossi et al. 2009), and in some cases are capable of detecting as few as three parasites in a single sand fly (Aransay et al. 2000, Michalsky et al. 2002). Traditional PCR equipment has become cheaper, lighter, and easier to use; however, requirement for gels, multi-step procedures, and the risk of contamination still preclude routine use under field conditions. The development of fluorogenic or real-time PCR assays has overcome many of the limitations of traditional PCR and offers great potential for use during military deployments. Real-time PCR assays have recently been used to detect *Leishmania* spp. parasites in sand flies in Italy (Gómez-Saladn et al. 2005) and Iraq (Coleman et al. 2006).

We have previously described the general situation that the U.S. military encountered at Tallil Air Base (TAB), Iraq, in March 2003 and discussed the factors that led to the establishment of a Leishmaniasis Control Program by the 520th Theater Army Medical Laboratory (TAML), the Army 787th Preventive Medicine Detachment, and the Public Health Section of the 407th Expeditionary Medical Support Unit (Coleman et al. 2004, 2005, 2006). We subsequently described the general biology of phlebotomine sand flies at TAB to include species diversity and temporal and geographic distribution of the sand flies (Coleman et al. 2007), as well as an evaluation of methods for the collection of sand flies (Burkett et al. 2007). In this study, we report our efforts to detect and identify *Leishmania* parasites in sand flies collected at TAB.

Materials and Methods

Collection of Sand flies. Methods used for the collection of sand flies have been described previously (Coleman et al., 2006, 2007). In brief, sand flies were collected in unbaited CDC miniature light traps (model 512; John W. Hock Company, Gainesville, FL) beginning in April 2003 and continuing thru October 2004. Traps were placed at 1800 hours local time and collected by 0800 hours the following day. On return to the field laboratory, collection cups containing the sand flies were placed in a −70°C freezer to kill the sand flies. Sand flies were separated by sex, and the numbers of male and female sand flies were recorded. Ten percent to 15% of the female sand flies and 95% of the male sand flies were placed in cryotubes containing 70% ethanol and shipped to the WRAIR where randomly selected specimens were identified to species. Eighty-five percent to 90% of the female sand flies and 5% of the male sand flies (included as negative controls) were placed in pools of 1–20 (separated by sex) in cryotubes containing 100% ethanol for subsequent testing for *Leishmania* parasites using a real-time (fluorogenic) PCR assay. These samples were stored at −70°C until tested.

DNA Extraction. The QIAamp DNA Mini Kit (catalog 51306; Qiagen, Valencia, CA) was used to extract DNA from pools of sand flies. Procedures used were as follows. Ethanol from each vial was removed with a pipette, and 180 μl of ATL buffer was added to each vial. Sand flies were triturated using a mortar and pestle until no discernible parts were visible. Twenty microliters of Proteinase K was added to each tube, and the tube was vortexed for 15 s and incubated at 56°C for 12 h. The tube was briefly centrifuged to collect the liquid into the bottom of the tube, and 200 μl of AL buffer was added, followed by vortexing for 15 s and incubation for 10 min at 70°C. Two hundred microliters of 100% ethanol was added, and the tube was briefly vortexed and centrifuged, after which the contents of each tube were placed in a QIAamp Spin Column and centrifuged at 8,000 rpm for 2 min in a microcentrifuge. The QIAamp Spin Column was

placed in a clean 2-ml collection tube, and 500 μl of AW1 buffer was added followed by centrifugation at 8,000 rpm for 2 min in a microcentrifuge. The QIAamp Spin Column was placed in a clean 2-ml collection tube, and 500 μl of AW2 buffer was added, followed by centrifugion at 13,200 rpm for 3 min in a microcentrifuge. The QIAamp Spin Column was placed in a clean 2-ml collection tube, and 50 μl of AE buffer was added, followed by incubation at room temperature for 5 min and centrifugion at 8,000 rpm for 1 min in a microcentrifuge. An additional 50 μl of AE buffer was added to the QIAamp Spin Column, followed by incubation at room temperature for 5 min and centrifugion at 8,000 rpm for 1 min in a microcentrifuge. Each tube containing ≈100 μl of sample was labeled and stored at 4°C if the PCR was to be performed within 3 d or at −20°C if the PCR was to be performed >3 d later.

Real-time PCR Assays. Samples were initially tested using a *Leishmania*-generic real-time PCR assay developed by Wortmann et al. (2001). The assay was modified so that each reaction contained one puReTaq Ready-to-Go PCR bead (Amersham Biosciences, Piscataway, NJ), 6 mM $MgCl_2$, 800 nM of each primer (LEIS L1, LEIS U1), 120 nM of probe (LEIS P1), and 2.0 μl of template DNA. Samples with a mean cycle threshold (Ct) value of 40 were considered negative. Approximately 10% of negative samples were randomly selected and retested using the same real-time PCR assay. Samples with a mean Ct value of <40.0 were considered potentially positive and were retested at least one additional time. Samples that tested positive the second time were considered "presumptive positives" (positive both times tested), whereas samples that tested negative the second time were tested a third time. Samples testing positive on this third test (positive two of three times tested) were considered "presumptive positives," whereas samples that tested negative the third time (positive one of three times tested) were considered indeterminate (i.e., could not determine whether they were true positives or true negatives). A summary of the algorithm for determining the infection status of the sand flies is presented in Fig. 1.

Three separate laboratories conducted real-time PCR testing including the TAML, an Air Force Biological Assessment Team (BAT), and the WRAIR. The TAML and the BAT were located at TAB, whereas the WRAIR is located in Silver Spring, MD. Although the same *Leishmania*-generic real-time PCR assay described above was used by each of the three laboratories, the assay was run using a different instrument at each laboratory including a Smartcycler used by WRAIR, a Lightcycler (Roche Diagnostics, Basel, Switzerland) used by the TAML, and an R.A.P.i.d. used by the BAT.

Conventional PCR. Samples that tested positive for *Leishmania* DNA using the genus-specific real-time PCR assay were further characterized using conventional PCR. Two markers were chosen to characterize and differentiate *Leishmania* species: glucose-6-phosphate-isomerase (GPI) and a putative intergenic spacer informally named "Hyper."

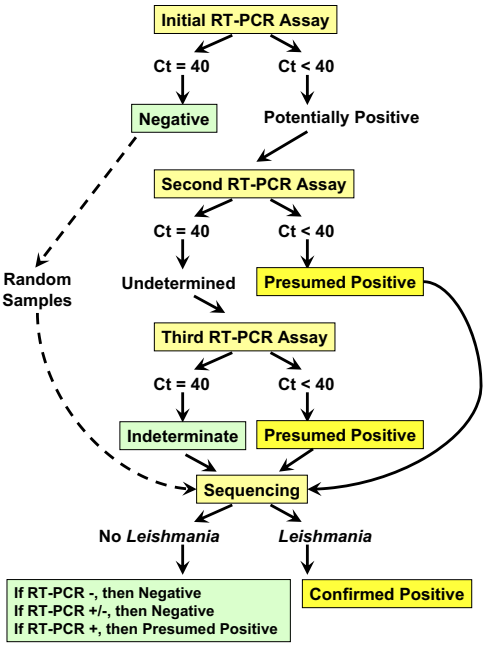

Fig. 1. Summary of the process used to detect and identify *Leishmania* parasites present in sand flies collected at Tallil Air Base, Iraq.

A 360-bp fragment of GPI was amplified. A first-round PCR primer set and a second-round nested PCR primer set were selected based on the only *Leishmania* GPI sequence (accession number X78206) contained in GenBank at the time of the primer design. The four primers used included LM21 F (exterior forward: 5′-GCA AGA CAT TCA CTA CAC AGG-3′), LM24R (exterior reverse: 5′-GGT GAA TGA GCT GGT AGA ATG-3′), LM22 F (interior forward: 5′-CGT TGC CCT ATC GAC CAA CAC-3′), and LM23R (interior reverse: 5′-CCT TTG CCG TTG CTC TCC ATG-3′). The first and second round PCR reactions were conducted as follows. Each 25-μl reaction contained one puReTaq Ready-to-Go PCR bead (Amersham Biosciences, Piscataway, NJ), 10 pmol of each primer, and 2 μl of template DNA, either *Leishmania* DNA as purified above or 2 μl of the first round PCR reaction. Cycling conditions for all reactions included an initial denaturation at 94°C for 2 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 45 s, with a final extension at 72°C for 7 min. Reactions were performed using an MJ-Research PTC-100 Thermal Cycler (MJ Research, Watertown, MA). Appropriate negative (water) and positive controls were included in all reaction sets. Positive samples were verified on a 1.5% agarose gel containing ethidium bromide by visualization of a band of the expected size using a transilluminator.

"Hyper" seems to be an intergenic region flanked upstream by a gene encoding a GTP-binding protein

Table 1. Evaluation of sand flies for *Leishmania* parasites using a *Leishmania*-generic real-time PCR assay

Testing unit[a]	Negative pools			Indeterminate pools			Positive pools			All pools	
	No. pools	Mean flies per pool (SD)	Ct (SD)	No. pools	Mean flies per pool (SD)	Ct (SD)	No. pools	Mean flies per pool (SD)	Ct (SD)	No. pools	Mean flies per pool (SD)
WRAIR	1,315	8.7 (5.41)	40 (-)	80	10.5 (4.38)	39.1 (1.03)	445	12.6 (3.69)	30.6 (4.80)	1,840	9.7 (5.27)
TAML	542	13.4 (3.86)	40 (-)	29	12.6 (5.42)	37.8 (1.26)	129	14.2 (2.91)	29.6 (4.65)	700	13.5 (3.79)
BAT	137	11.6 (4.79)	40 (-)	0	N/A		36	14.8 (0.96)	29.2 (3.60)	173	12.3 (4.47)
All	1,969	10.1 (5.42)	40 (-)	80	10.5 (4.38)	38.6 (0.93)	456	12.7 (3.67)	30.3 (4.69)	2,505	10.6 (5.20)

[a] One hundred eighty pools were tested by both WRAIR and the TAML, and 28 pools were tested by both WRAIR and the BAT.
Ct, cycle threshold.

and downstream by a gene encoding a protein of unknown function in *L. braziliensis* (Fu et al. 1998), *L. infantum* (Peacock et al. 2007), *L. major* (Ivens et al. 2005), and several other *Leishmania* species (Moulton et al., unpublished data). The entire spacer region was amplified and sequenced using primers positioned at the 3′ end of the GTP gene (5′-GCACGAGGACATCTGCCTCG-3′) and the 5′ end (5′-GCTTGCGTGCCTCGGGGTACTT-3′) of the downstream gene. Comparisons between sequences from a handful of strains of both *L. donovani* and *L. infantum* (including a putative *L. chagasi*) showed no intraspecific polymorphism and 10 putative species-specific differences: 9 point mutations and a single nucleotide insertion/deletion. The ≈600-bp region sequenced in this study was amplified using visceral *Leishmania*-specific primers (5′-CGTGCAGTGGCGCAG-3′ and 5′-YGCTCGAACAAAAAGM-3′) and possesses four substitution (all transitions) differences and one point deletion/insertion event between *L. donovani* and *L. infantum/chagasi* (Moulton et al., unpublished data).

DNA Sequencing. All samples for which GPI was successfully amplified using conventional PCR were either sequenced at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) at Ft. Detrick, MD, or under commercial contract to Agencourt Biosciences (Beverly, MA). In brief, procedures for sequencing were as follows. The PCR amplification product remaining after gel electrophoresis was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. Automated sequencing was performed using an ABI 3100 genetic analyzer and a Big-Dye v1.1 or v3.1 sequencing kit (PE Biosystems, Foster City, CA) according to the manufacturer's instructions. Primer, excess nucleotides, and buffer were removed from the Big-Dye sequencing reaction by eluting the material from a Sephadex G-50 (Amersham Biosciences) column equilibrated with water.

Sequencing of "Hyper" was conducted at the University of Tennessee and differed procedurally as follows: PCR products were electrophoresed in 1% agarose, excised from the gel, and purified using a QiaQuick Gel Extraction Kit (Qiagen); sephadex-cleaned sequencing reactions were electrophoresed through a 6% polyacrylamide gel using an MJ Research BaseStation 100 Automated DNA Sequencer (Bio-Rad, Hercules, CA) and analyzed using Cartographer 1.2.7 software; and sequences from opposing strands were reconciled and verified for accuracy using Sequencher 4.2.2 (Gene Codes, Ann Arbor, MI).

Phylogenetic Analysis. GPI sequences were aligned using the MegAlign program (Lasergene analysis software; DNASTAR, Madison, WI), and sequence ends were trimmed to a uniform length. Phylogenetic analyses of aligned sequences were performed using the ClustalW method (Higgins et al. 1996) with a gap penalty of 15 and a gap length of 6.66. The phylogenetic tree generated by MegAlign is a rooted tree with the number of substitution events indicated at the bottom of the tree. Bootstrap replication was used to evaluate the strength of the clustering analysis. Unknown sample sequences were compared with sequences determined for known culture isolates and to other sequences present in GenBank.

Results

***Leishmania*-generic Real-time PCR Assay.** A total of 2,505 pools containing 26,574 sand flies collected at Tallil Air Base was tested using a *Leishmania*-generic real-time PCR assay (Table 1). These pools included 2,479 pools containing 26,177 female sand flies and 26 pools containing 397 male sand flies (included as negative controls). The species of sand flies contained in each pool were not determined; however, prior studies indicated that *Phlebotomus papatasi* Scopoli, *Phlebotomus alexandri* Sinton, and *Sergentomyia* spp. were the predominant sand flies collected at TAB (Coleman et al. 2007). The TAML and the BAT tested 700 and 173 pools, respectively, in Iraq, whereas 1,840 pools were shipped to the United States and were tested at the WRAIR in Silver Spring, MD. The pools tested at the WRAIR included 180 pools that had been previously tested by the TAML and 28 that had been tested by the BAT.

A total of 1,969 pools (78.6% of all pools tested) were negative for *Leishmania* DNA (Table 1), including 1,943 pools containing 19,569 female sand flies and all 26 pools containing 397 male sand flies. A randomly selected sample of 192 (9.8%) negative pools was retested using the same real-time PCR assay—all of these pools were negative on retesting. A total of 536 pools (21.4% of all pools tested) containing 6,608 female sand flies tested positive for *Leishmania* DNA using the real-time PCR assay. Each of these pools was subsequently retested. After all retesting was completed, a total of 456 pools were considered positive,

Table 2. Comparison of real-time PCR assay results for samples that were tested on different PCR platforms at different laboratories

Lab[a]	Initial test laboratory results			WRAIR results				Correlation R^{2c}
	Initial result	No. samples	Mean Ct[b] (SD)	Mean Ct (SD)	No. negative (%)	No. indeterminate (%)	No. positive (%)	
BAT	Positive	28	28.64 (3.62)	30.49 (3.91)	0 (0)	0 (0)	28 (100)	0.86
TAML	Negative	25	40.00 (0.00)	40.00 (0.00)	25 (100)	0 (0)	0 (0)	1.00
TAML	Indeterminate	31	38.11 (1.27)	38.66 (2.24)	19 (61)	4 (13)	8 (26)	0.05
TAML	Positive	124	30.88 (5.08)	29.45 (4.42)	7 (6)	3 (2)	114 (92)	0.84

[a] The PCR platforms used by the BAT, TAML, and WRAIR were the Idaho Technology RAPID, the Roche Lightcycler, and the Cepheid Smartcycler, respectively.
[b] The Ct value is the no. of cycles at which the sample was considered positive. A lower Ct value would correlate with more *Leishmania* DNA present in a sample. A Ct value of 40 is considered negative.
[c] Pearson correlation.

and 80 pools were considered indeterminate (Table 1). Assuming that only a single sand fly in each pool was positive, the minimum field infection rate was 2.33% (456/19,569).

In addition to the above studies, we also tested a total of 290 individual sand flies that had been identified to species (*P. papatasi*, *P. alexandri*, or *P. sergenti*) or genus (*Sergentomyia*). Four of 114 (3.5%) *P. alexandri* tested positive using the *Leishmania*-generic real-time PCR assay. Unfortunately, we were unable to determine the species of parasite using either sequencing or a real-time PCR assay specific for parasites that cause visceral leishmaniasis. All other individual sand flies, to include 77 *Sergentomyia* spp., 94 *P. papatasi* and five *P. sergenti*, were negative when tested using the real-time PCR assay.

Agreement Between Real-time PCR Results Obtained by Different Laboratories. Because three separate laboratories were involved in the testing of sand flies, we evaluated agreement between PCR results for all samples that were tested at multiple laboratories (Table 2). A total of 180 samples were tested by both the WRAIR and the TAML and 28 by the WRAIR and the BAT. All 28 samples tested initially by the BAT using the R.A.P.i.d. and subsequently by the WRAIR using the Smartcycler were positive for *Leishmania* at both laboratories. Although there were significant differences (paired-samples *t*-test: $P < 0.001$, $t = 4.819$, df = 27) in the mean Ct values obtained by the two laboratories; there was a strong positive correlation ($r^2 = 0.86$, Pearson's correlation) between the results obtained by the two laboratories (Table 2).

Of the 180 samples initially tested by the TAML using the Lightcycler, 25 were considered negative, 124 were positive, and 31 were indeterminate (Table 2). All 25 negative samples were also negative when retested by the WRAIR. Of the 124 samples that were considered positive when tested by the TAML, 114 (92%) were also considered positive when retested by the WRAIR; however, 7 (6%) were considered negative and 3 (2%) were indeterminate. Although there were significant differences (paired-samples *t*-test: $P < 0.001$, $t = 5.836$, df = 123) in the mean Ct values obtained by the two laboratories; there was a strong positive correlation ($r^2 = 0.84$, Pearson's correlation) between the results obtained by the two laboratories (Table 2). Of the 31 samples considered indeterminate by the TAML, 19 (61%) were negative when retested by the WRAIR, whereas 8 (26%) were considered positive and 4 (13%) were considered indeterminate. There was a poor correlation ($r^2 = 0.05$, Pearson's correlation) in the results obtained by the two laboratories for these samples.

Analysis of the GPI Gene. A total of 35 known species/strains of *Leishmania* served as references for identification and phylogenetic analysis of *Leishmania* DNA extracted from pools of sand flies (Table 3). In

Table 3. *Leishmania* strains used as positive controls for PCR and sequencing reactions

Leishmania species	Strain	Infectious to humans	Disease caused
L. chagasi	PP-75	Yes	VL
L. donovani	DD8	Yes	VL
L. donovani	LV9	Yes	VL
L. donovani	LV9a	Yes	VL
L. infantum	LP49D	Yes	VL
L. infantum	LP53D	Yes	VL
L. infantum	LP59H	Yes	VL
L. infantum	UA 973	Yes	VL
L. mexicana	UA 1182	Yes	CL
L. mexicana	UA 2336	Yes	CL
L. major	CSU	Yes	CL
L. major	Jericholl	Yes	CL
L. major	LRC 490	Yes	CL
L. major	LRC 496	Yes	CL
L. major	Pt1	Yes	CL
L. major	Pt3	Yes	CL
L. major	Pt4	Yes	CL
L. major	WR2551	Yes	CL
L. major	WR2580	Yes	CL
L. major	ATCC 50122	Yes	CL
L. major	ATCC 50123	Yes	CL
L. tropica	EP40	Yes	CL
L. tropica	EP81	Yes	CL
L. tropica	EP82	Yes	CL
L. tropica	EP87	Yes	CL
L. tropica	EP97	Yes	CL
L. tropica	EP100	Yes	CL
L. tropica	LRCL	Yes	CL
L. tropica	LRC L590	Yes	CL
L. tropica	WR1063	Yes	CL
L. tarentolae	ATCC 30143	No	—
L. adleri	LV30	No	—
L. braziliensis	UA 859	Yes	MC
L. gerbilli	ATCC 50121	No	—
L. aristedsi	TCC 50124	No	—

VL, visceral leishmaniasis; CL, cutaneous leishmaniasis; MC, mucocutaneous leishmaniasis.

Table 4. Initial analysis of the GPI gene[a] from sand flies that were positive, negative, or indeterminate for *Leishmania* DNA using a *Leishmania*-generic real-time PCR assay

PCR result	Sequencing result				Total
	Leishmania GPI	*Anopheles*-like GPI	Other GPI	Negative	
Negative	0 (0.0%)	12 (92.3%)	0 (0.0%)	1 (7.7%)	13
Indeterminate	3 (3.8%)	67 (83.8%)	7 (8.8%)	3 (3.8%)	80
Positive	281 (64.0%)	153 (34.9%)	1 (0.2%)	4 (0.9%)	439
Total	284 (53.4%)	232 (43.6%)	8 (1.5%)	8 (1.5%)	532

[a] Three hundred sixty–base pair region of the GPI gene.

addition, DNA from three species of phlebotomine sand flies *Lutzomyia longipalpis* (Lutz and Neiva), *P. papatasi*, and *Phlebotomus argentipes* (Annandale and Brunetti) that had been colonized at the WRAIR served as negative controls. These laboratory-reared sand flies repeatedly tested negative for the presence of *Leishmania* DNA using the real-time PCR assay. A conventional PCR assay that was designed to amplify a 360-bp section of the GPI gene was used to generate PCR products for sequencing from 532 pools of field-collected sand flies, to include 13 negative pools, 439 *Leishmania*-positive pools, and 80 *Leishmania*-indeterminate pools (using the real-time PCR assay; see Table 4). Seventeen of the 456 samples that were considered positive after real-time PCR analysis were not available for sequencing, primarily because of insufficient sample.

PCR amplification and subsequent sequencing were successful for 524 (98%) of the 532 pools evaluated (Table 4). *Leishmania* GPI was detected in 284 (53%) of the pools, including 281 (64%) of the 439 PCR-positive samples and 3 (4%) of the 80 PCR-indeterminate samples. *Leishmania* GPI was not detected in any of the 13 PCR-negative samples or in any of the laboratory-colonized sand flies. Although the PCR amplifications and sequencing were successful for the remaining 240 samples, the sequences did not match any known *Leishmania* sequences. However, 232 of these sequences had >80% homology to the GPI gene from *Anopheles gambiae* str. PEST (GenBank accession no. XM 320366), and 8 were a close match to other non-*Leishmania* GPI sequences. The *Anopheles*-like sequences were detected in 92% (12/13) of the PCR-negative pools, in 84% (67/80) of the PCR-indeterminate pools, and in 100% (6/6) of the laboratory-colonized sand flies; however, these sequences were only detected in 35% (153/439) of the real-time PCR-positive pools (Table 4).

A phylogenetic comparison of the different sequences determined for the *Leishmania* parasites detected in the sand fly pools is presented in Fig. 2. The species identities of the field samples were determined based on a cluster analysis and comparison to the sequences determined for known *Leishmania* parasites. A representative sampling of the *Anopheles*-like sequences is included in this cluster analysis. Of the 284 sequences identified as *Leishmania*, only 23 were from medically important species: 18 (6.3%) were *L. donovani*-complex parasite sequences, 3 (1.1%) were *L. tropica* (Wright) sequences, and 2 (0.7%) contained sequences that were similar to both *L. major* and *L. tropica* (Table 5). These latter parasites are henceforth referred to as *L. major/tropica*-like. Minimum field infection rates (assuming a single infected fly per positive pool) were 0.09% for *L. donovani*-complex parasites, 0.02% for *L. tropica*, and 0.01% for the *L. major/tropica*-like parasites. The overwhelming majority (261; 91.9%) of isolates were identified as *L. tarentolae* Wenyon, a *Leishmania* parasite normally associated with gecko lizards (Wallbanks et al. 1985, Elwasila 1988).

Because of the fact that many of the samples that tested positive for *Leishmania* DNA using the *Leishmania*-generic real-time PCR assay failed to yield *Leishmania*-GPI sequence data, we compared the real-time PCR Ct values against the sequencing data obtained for the same sample (Table 6). As the mean Ct values increased (weaker reaction), the proportion of samples that resulted in the identification of *Leishmania*-GPI sequences decreased, whereas the proportion of other non-*Leishmania*-GPI sequences increased. For example, 99% of the samples with a mean Ct value of <26 matched known *Leishmania* spp. sequences, whereas only 3% of the samples with a mean Ct value of ≥38 matched any known *Leishmania* spp. sequences (Table 6).

Analysis of the "Hyper" Gene. Because analysis of the GPI gene could not differentiate between *L. donovani* and *L. infantum*, we subsequently sequenced a 600-bp region of the intergenic region "Hyper" from 12 samples that sequencing of the GPI gene had identified as *L. donovani*-complex parasites. Sequencing of the "Hyper" gene indicated that all 12 of these samples contained *L. infantum*, but not *L. donovani*, as evidenced by the presence of all five putative *L. infantum*–specific mutations (four substitutions, one point deletion/insertion) and no *L. donovani*–specific mutations, either alone or in conjunction with those of *L. infantum* (i.e., polymorphisms would indicate co-presence).

Temporal Distribution of *Leishmania*-infected Sand Flies. Figure 3 presents the temporal distribution of all *Leishmania*-infected sand flies as determined by the *Leishmania*-generic real-time PCR assay and shows the individual distribution for sand flies infected with *L. donovani*-complex parasites, *L. major/tropica*-like parasites, and *L. tarentolae* parasites as determined by sequencing of the GPI gene. Infected sand flies were collected as early as late April/early May 2003, with the first PCR-positive sand fly col-

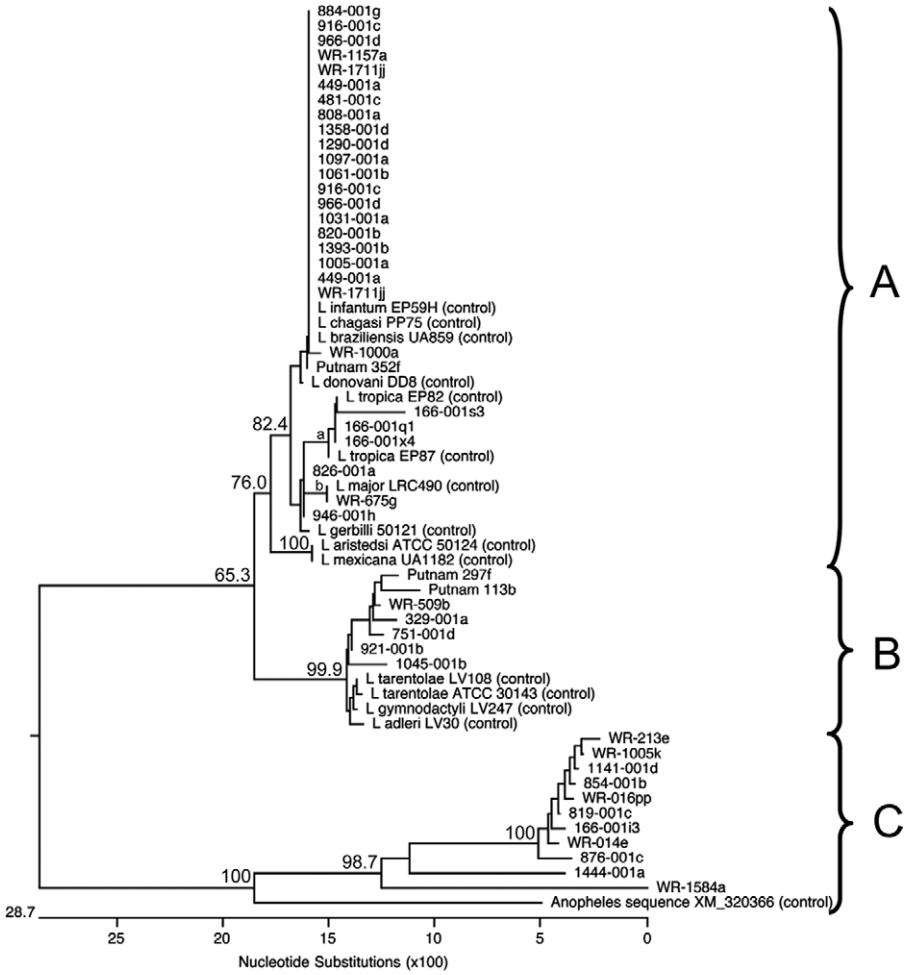

Fig. 2. Phylogenetic tree based on the alignment of a 360-bp fragment of the glucose 6-phosphate isomerase gene from *Leishmania* spp. amplified from sand flies collected at Tallil Air Base, Iraq. Duplicate sample sequences have been removed to condense the tree. Samples in bold are field isolates; all others are known culture isolates. (A) Medically important *Leishmania* spp., *L. infantum*, *L. major*, *L. tropica*, *L. donovani*, *L. chagasi*, and *L. mexicana*. (B) Nonmedically important *Leishmania* spp., *L. tarentolae*. (C) *Anopheles* GPI-like and other GPI sequences.

lected on 23 April 2003 and the first sequence-positive sand fly detected on 3 May 2003. Overall infection rates, as determined by the *Leishmania*-generic real-time PCR assay, rose from 1.2% in April and May to a high of 2.3% in July, followed by a decrease in August and September. Infection rates for *L. tarentolae* were similar to the overall real-time PCR positive rates except that they were lower and that no positive pools were detected in April. Infection rates for *L. donovani*-complex parasites were ≈10-fold lower than that determined for *L. tarentolae*, with positive sand flies collected from May throughAugust. Sand flies infected with *L. tropica* and *L. major*/*tropica*-like parasites were only collected in May and June.

Table 5. Detailed analysis of the GPI gene[a] from 284 samples initially identified as positive for *Leishmania* parasite DNA using a *Leishmania*-generic real-time PCR assay

Sequence results	Real-time PCR-positive samples	Real-time PCR indeterminate samples	Minimum infection rate (# positive pools/total # sand flies)
L. donovani-complex	17 (6.04%)	1 (33.33%)	0.09% (18/19,569)
L. major/*tropica*-like	2 (0.71%)	0	0.01% (2/19,569)
L. tropica	2 (0.71%)	1 (33.335%)	0.02% (3/19,569)
L. tarentolae	260 (92.53%)	1 (33.33%)	1.33 (261/19,569)
All *Leishmania*	281 (100%)	3 (100%)	1.45% (284/19,569)

[a] Three hundred sixty–base pair region of the GPI gene.

Table 6. Relationship between the cycle threshold (Ct) values for the *Leishmania*-generic real-time PCR assay and the proportion of samples determined to be positive on retesting using the same PCR assay and by sequencing of a portion of the GPI gene

Ct value	Real-time PCR assay results				Sequencing results		
	No. samples	No. (%) negative[a]	No. (%) positive[a]	No. (%) indeterminate[a]	No. sequenced	No. (%) *Leishmania*	No. (%) other
40	1,969	1,969 (100%)	0 (0%)	0 (0%)	12	0 (0%)	13 (100%)
38–39.99	75	0 (0%)	3 (4%)	72 (96%)	74	3 (4%)	71 (96%)
36–37.99	76	0 (0%)	69 (91%)	7 (9%)	76	8 (11%)	68 (89%)
34–35.99	52	0 (0%)	52 (100%)	0 (0%)	51	11 (22%)	40 (78%)
32–33.99	56	0 (0%)	55 (98%)	1 (2%)	47	19 (40%)	28 (60%)
30–31.99	68	0 (0%)	68 (100%)	0 (0%)	66	47 (71%)	19 (29%)
28–29.99	60	0 (0%)	60 (100%)	0 (0%)	59	52 (88%)	7 (12%)
26–27.99	48	0 (0%)	48 (100%)	0 (0%)	45	44 (98%)	1 (2%)
<26	101	0 (0%)	101 (100%)	0 (0%)	101	100 (99%)	1 (1%)
Total	2,505	1,969 (79%)	456 (18%)	80 (3%)	531	284 (53%)	248 (47%)

[a] The determination as to whether the initial real-time PCR assay result was positive, negative, or indeterminate was based on retesting of the sample using the same real-time PCR assay, as outlined in Figure 1.

Geographic Distribution of *Leishmania*-infected Sand Flies. The geographic distribution of sand flies infected with *L. donovani*-complex parasites, *L. tropica* parasites, and *L. major/tropica*-like parasites is presented in Fig. 4. The 18 pools that contained *L. donovani*-complex parasites were collected at nine different locations, including several areas where high densities of U.S. military personnel were located. Infection rates at the different locations ranged from a low of 0.04% to a high of 0.43%. The combination of the temporal and the geographic data suggests that *L. donovani*-complex parasites are widely distributed at TAB.

Interestingly, all three pools of sand flies in which *L. tropica* was detected were collected from the same light trap on 3 May 2003 (Fig. 4). Because this was somewhat surprising, a more thorough evaluation of the data are provided for the sand flies that were collected in this trap. This trap was placed ≈5 m from a house and was not located near any caves or burrows of potential reservoir animals. Of the 700 sand flies collected on 3 May, 625 were separated into 91 pools and tested using the real-time PCR assay. Thirteen of the 91 pools initially tested positive for *Leishmania.* However, after retesting, five pools were considered presumptive positives, whereas eight were considered indeterminate. Samples for all 13 of these pools were subsequently sequenced. *L. tropica* was detected in three of the five presumptive positive pools and *L. tarentolae* in a fourth presumptive positive pool. *Leishmania*-GPI sequences were not detected in any of the eight indeterminate pools or in one of the five presumptive positive pools. Although all three *L. tropica*–positive pools were detected during the same real-time PCR assay run, the first positive sample was separated from the second positive sample by 6 negative samples, whereas the second positive sample was separated from the third positive sample by 19 nega-

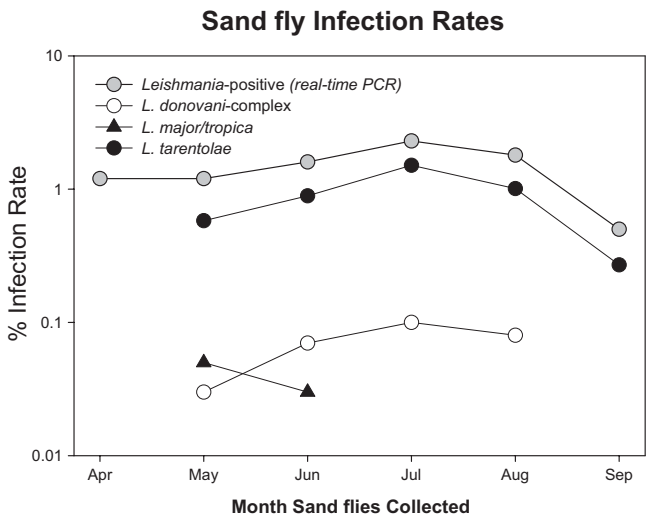

Fig. 3. Infection rates of phlebotomine sand flies infected with *Leishmania* spp. parasites as listed by month at Tallil Air Base. Rates for *L. donovani*-complex, *L. major/tropica*-like, and *L. tarentolae* parasites were based on sequencing of a 360-bp region of the glucose-6-phosphate isomerase gene, whereas overall *Leishmania*-positive rates were based on the *Leishmania*-generic real-time PCR assay.

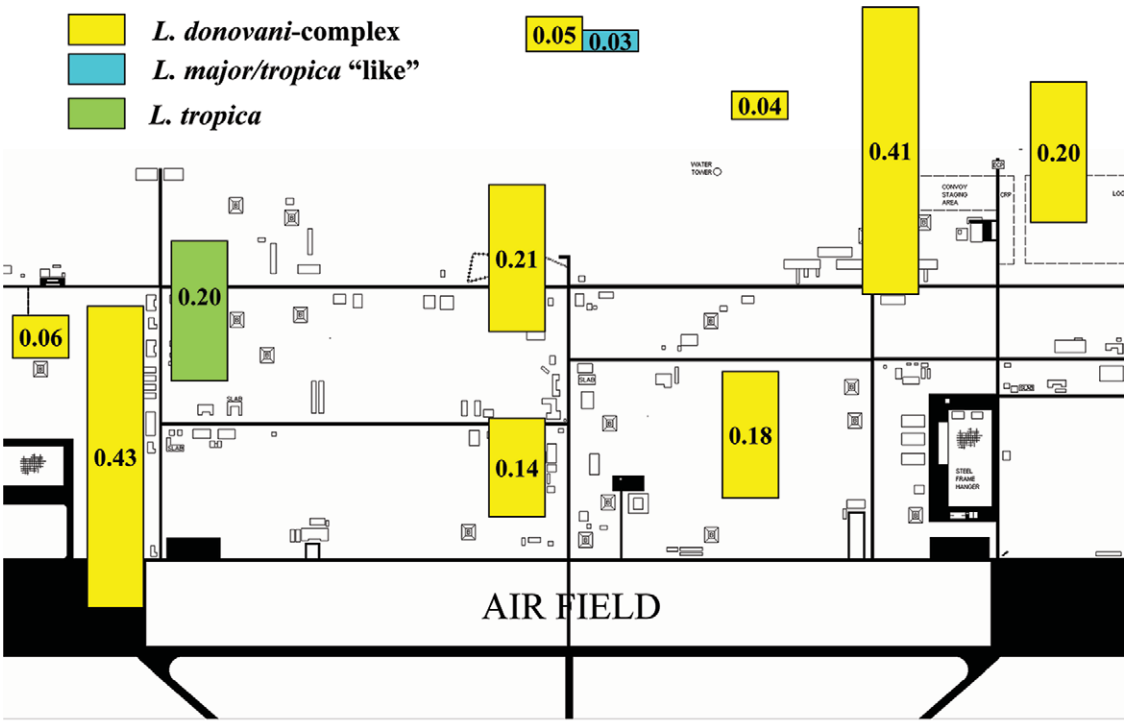

Fig. 4. Distribution of *L. donovani*-complex, *L. tropica*, and a *L. major/tropica*-like parasite at Tallil Air Base, Iraq. The height of the bar corresponds to the percent of sand flies that tested positive for the indicated *Leishmania* spp.

tive samples. These data suggest that these were true positive pools and that they did not result from the cross-contamination of samples.

Both sand fly pools that contained the *L. major/tropica*-like GPI sequences were collected from the same light trap; however, one sample was collected on 21 June 2003 and one on 30 June 2003 (Fig. 4). One of two pools collected on 21 June and one of 19 pools collected on 30 June tested positive by both real-time PCR and sequencing for the *L. major/tropica*-like GPI sequences. The fact that the two *L. major/tropica*-like samples were detected on different days and that a number of samples adjacent to both of these positive samples were negative suggests that both of these samples represent true positives and that they were not the result of cross-contamination during either the DNA extraction process or the real-time PCR procedure.

The geographic distribution of *L. tarentolae*–infected sand flies is presented in Fig. 5. *L. tarentolae* was detected in sand flies collected throughout TAB, with infection rates ranging from a low of 0.12% to a high of 2.61%. Sand flies infected with *L. tarentolae* were not collected at the site where the *L. tropica*--infected sand flies were collected; however, they were collected in all locations where sand flies infected with *L. major* and *L. donovani*-complex parasites were found.

Discussion

In this study, we provided the first in-depth examination of *Leishmania* infection rates in sand flies collected at Tallil Air Base, Iraq. Although our data have direct relevance for those military personnel who were actually stationed at TAB, our conclusions may have a bearing on all military personnel deployed to Iraq.

Visceral Leishmaniasis at TAB. Our data showed that parasites that cause visceral leishmaniasis (e.g., *L. donovani*-complex parasites) posed a threat to military personnel stationed at TAB in 2003 and 2004. Our data also showed that *L. infantum* s. str. was the only *L. donovani*-complex parasite detected at TAB. Although the overall minimum field infection rate for the *L. donovani*-complex parasites was only 0.09%, the rate was much higher at certain sites and at certain times of the year. For example, at one site, the overall rate for 2003 and 2004 was 0.43% (Fig. 4); however, the rate for July 2003 was 1.0% (2/198). At another site, the overall rate was 0.41%; however, the rate for May and June 2003 was 1.43% (1/70) and 1.64% (1/61), respectively. Although our procedures did not allow us to determine whether these infected sand flies were capable of transmitting *L. donovani*-complex parasites or whether they were feeding on humans, a number of facts suggest that individuals living at TAB were at risk.

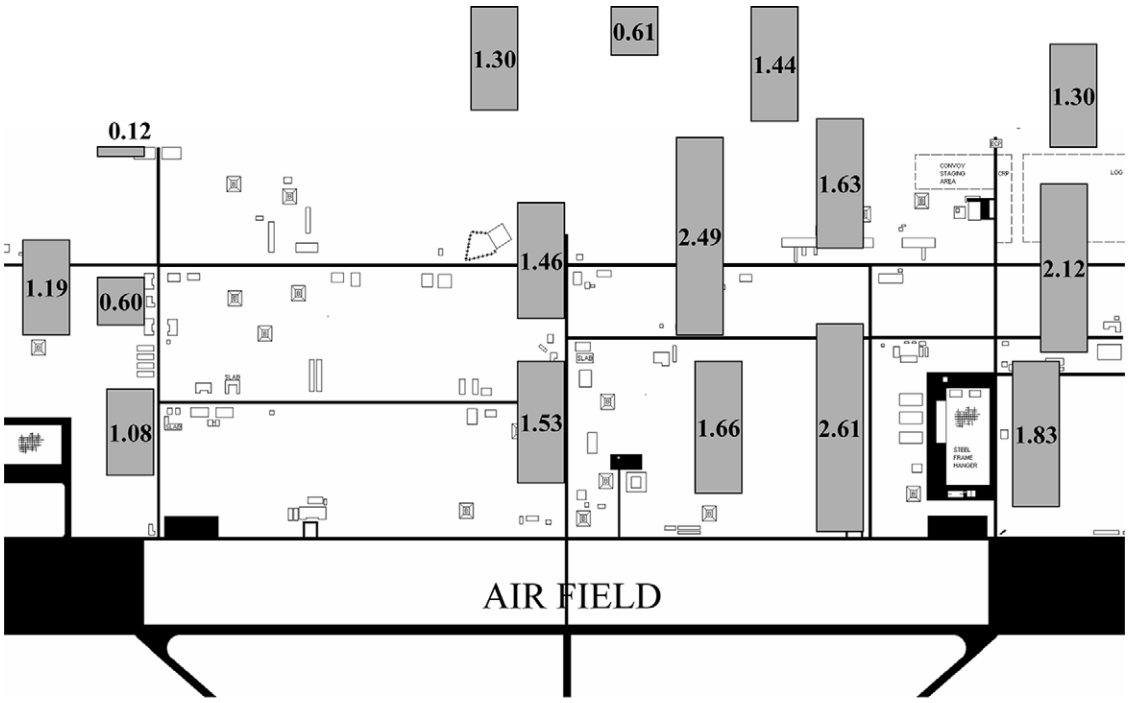

Fig. 5. Distribution of *L. tarentolae* at Tallil Air Base, Iraq. The height of the bar corresponds to the percent of sand flies that tested positive for *L. tarentolae*.

These facts include the following: (1) as many as 1 of every 61 flies collected at certain sites/times at TAB was infected with *L. donovani*-complex parasites; (2) many military personnel living in areas where *L. donovani*-complex parasites were detected reported being bitten by sand flies, with some units reporting up to 75% of the soldiers receiving up to 1,000 bites in a single night (Coleman et al. 2006); (3) *Phlebotomus alexandri*, a suspected vector of *L. infantum* in the Middle East (Azizi et al. 2006), was the primary species of sand fly collected at TAB and accounted for 30% of all specimens collected (Coleman et al. 2007); (4) *P. alexandri* is known to readily feed on humans (Azizi et al. 2006); and (5) many canids, including feral and semidomesticated dogs and golden jackals (*Canis aureas* L.), were found throughout TAB and could have potentially served as disease reservoirs (Gramiccia and Gradoni 2005).

Historically, visceral leishmaniasis in Iraq was most commonly diagnosed in the Greater Baghdad area and was not considered a threat in the southern part of the country. However, since 1991, the disease has been reported in areas where it was previously unknown or very rare, to include the Thi-Qar Governate, the province where TAB is located (World Health Organization, 2003). Between 1989 and 2001, the reported incidence of visceral leishmaniasis in Iraq ranged from a low of 2.6 per 100,000 (491 total cases) in 1989 to a high of 20.0 per 100,000 (3,866 total cases) in 1992 (World Health Organization 2003). Iraqi physicians in An Nasiriyah, a small city located ≈20 km to the northeast of TAB, reported that visceral leishmaniasis caused by *L. infantum* has been a growing problem since the late 1990s, with several hundred children hospitalized each year (COL P. Weina, personal communication). Visceral leishmaniasis caused by *L. donovani* has not been reported in this area. This information suggests that the *L. donovani*-complex parasites identified in sand flies collected at TAB are likely *L. infantum* and not *L. donovani.* Our analysis of the "Hyper" gene of 12 of the 19 *L. donovani*-complex positive samples confirms that *L. infantum* was present at TAB.

To date, only three military personnel deployed to Iraq since 2003 have developed visceral leishmaniasis, with none of these three individuals known to have spent time at TAB. Nevertheless, we believe that dozens or even hundreds of individuals stationed at TAB may have been exposed to parasites that cause visceral leishmaniasis, recognizing that exposure does not constitute infection. The fact that more symptomatic cases have not yet occurred in deployed military personnel is not unexpected because visceral leishmaniasis caused by *L. infantum* has historically been considered a disease of young children who are malnourished and/or immunocomprimized (Fenech 1997). Iraqi physicians in An Nasiriyah reported that 95% of the cases of visceral leishmaniasis in the region

occur in malnourished children who are <1 yr of age, with 99% of the cases occurring in children <5 yr of age (COL P. Weina, personal communication). In the Mediterranean basin, symptomatic visceral leishmaniasis in adults is almost exclusively a result of concomitant infection with *L. infantum* and human immunodeficiency virus (Fenech 1997, Dedet and Pratlong 2000). The fact that deployed military personnel are presumably healthy, well-fed, immune-competent adults suggests that the risk of developing symptomatic visceral leishmaniasis is low; however, a number of factors suggest that there may be potential long-term concerns involving the disease. Although *L. infantum* primarily exists as an asymptomatic infection (Le Fichoux et al. 1999, Sakru et al. 2007), asymptomatic carriers can become symptomatic after suppression of their immune system (Dedet and Pratlong 2000). The high rate of asymptomatic *L. infantum* infections in the Mediterranean basin (Le Fichoux et al. 1999, Riera et al. 2004, Sakru et al. 2007) only became apparent as a result of an outbreak of symptomatic visceral leishmaniasis infections that resulted from concomitant HIV infections (Molina et al. 2003). The visceral leishmaniasis outbreak led to an increase in surveillance and detection of the asymptomatic visceral leishmaniasis infections. A variety of data suggest that *Leishmania* parasites can persist in the body for life, even after successful treatment of symptomatic individuals (Bogdan and Röllinghoff 1998). Morales et al. (2001) reported the detection of *L. infantum* in the blood of HIV-infected patients for several years, whereas Svobodova et al. (2003) found that asymptomatic laboratory rats harbored *L. tropica* for several years. These data suggest that individuals asymptomatically infected with parasites that cause visceral leishmaniasis at TAB could develop symptomatic leishmaniasis at a later date or, even if remaining asymptomatic, could serve as a reservoir capable of infecting other individuals through a variety of mechanisms. *Leishmania* parasites are a known threat to the blood supply (Cardo 2006) and can be transmitted through blood transfusions (Dey and Singh 2006). Although Colomba et al. (2005) found that none of 500 blood donors in Italy had anti-*Leishmania* antibodies, Riera et al. (2004) reported that sera from 7.6% of randomly selected blood donors in Spain tested positive by Western blot for parasites that cause visceral leishmaniasis, whereas Le Fichoux et al. (1999) found that sera from 76 of 565 (13.5%) blood donors in southern France contained *Leishmania*-specific antibodies, including nine serum samples (1.65%) that were positive by culture. In addition to the well-documented threat to the blood supply, a limited set of data suggests that *Leishmania* parasites can be transmitted congenitally in mice (Rosypal and Lindsay 2005), dogs (Rosypal et al. 2005), and humans (Eltoum et al. 1992, Meinecke et al. 1999). Riera and Valladares (1996) also showed the presence of viable *L. infantum* parasites in the urine and semen of experimentally infected dogs. In addition to the potential threat of person-to-person transmission through any of these mechanisms, asymptomatically infected military personnel could potentially infect sand flies present in the United States, although little information is available regarding the ability of North American sand flies to transmit *L. donovani*-complex parasites. Canine leishmaniasis caused by infection with *L. infantum* has emerged as a significant problem in the United States (Rosypal et al. 2003), yet the role of endemic sand flies in its maintenance and transmission has not been elucidated (Ostfeld et al. 2004). However, Svobodova et al. (2003) found that asymptomatic laboratory rats were capable of infecting sand flies for several years after being infected with *L. tropica.* To date, no comparable studies have been conducted with *L. infantum.*

Although it is impossible to determine what proportion of the infected sand flies at TAB was capable of transmitting *Leishmania*, the fact that sand flies infected with *L. donovani*-complex parasites were found throughout much of TAB suggests that the threat to military personnel may have been widespread (Fig. 4). Likewise, although the exact number of individuals actually exposed to parasites that cause visceral leishmaniasis may never be determined, because there are currently no FDA-licensed tests that can be used to assess exposure, these data suggest that undetected, asymptomatic visceral leishmaniasis may pose a future problem for U.S. military personnel who deployed to Iraq.

Cutaneous Leishmaniasis. To date, all but three of the >2,000 cases of leishmaniasis reported in U.S. military personnel deployed to Iraq since 2003 have been cutaneous leishmaniasis caused by *L. major* (COL P. Weina, personal communication). The majority of these cases occurred in personnel stationed along the Iranian border northeast of Baghdad or in the vicinity of Tal Afar in Northern Iraq. Although it is difficult to determine the exact number of cutaneous leishmaniasis cases that were acquired in the vicinity of TAB, the infectious diseases physician assigned to the TAML estimates that <25 cases of cutaneous leishmaniasis occurred at TAB (COL P. Weina, personal communication). Of the 284 pools of sand flies in which *Leishmania* parasites were confirmed by sequencing, only three pools were positive for *L. tropica* and two for a parasite that is similar to both *L. major* and *L. tropica.* Because *L. tropica* was only detected in sand flies collected from a single trap on 3 May 2003, this suggests that the parasite was much more focal than either the *L. donovani*-complex parasites or *L. tarentolae*, which were much more widely distributed temporally and geographically. Although we were not able to determine the species of sand fly that was infected with *L. tropica*, [mt35% (19/52) of all female *P. sergenti* identified during the course of this study were collected between 22 Apr and 10 May 2003, with most of these collected within 100 m of where the *L. tropica*–infected sand flies were captured. *Phlebotomus sergenti* is a known vector of *L. tropica* in the Middle East (Svobodova and Votýpka 2003) and has been found throughout Iraq (Sukkar 1974, Sukkar et al. 1985). The detection of *L. tropica* in sand flies collected in early May 2003 has prompted speculation regarding the mammalian reservoir that could have

served as the source of infection. Although humans are believed to be the primary reservoir of *L. tropica* in urban areas (Jacobson 2003), this is probably not the case at TAB because all Iraqi military and civilian personnel had vacated the base by 23 March 2003. Because sand flies were not active at TAB until mid-April, the explanation that adult sand flies had become infected with *L. tropica* after feeding on an infected Iraqi in 2002 seems unlikely, because phlebotomine sand flies are believed to overwinter as eggs and larvae and not as adults (Lawyer and Young 1991). It is more likely that sand flies became infected after feeding on an unknown animal. Although animals are believed to be the reservoir of *L. tropica* in rural areas, the full life cycle is still being studied (Jacobson 2003), and the reservoir in Iraq is not known. Interestingly, sequences for two of the three *L. tropica* isolates were 100% identical, with the third isolate being 70% identical to the other two. Because all three isolates were collected on the same day from the same trap, it is possible that the same reservoir may have infected two of the sand flies, whereas a different reservoir may have infected the third sand fly. The two *L. major/tropica*-like isolates were 100% identical, and the fact that the sand flies from both positive pools were collected from the same light trap, albeit on different days, suggests that both sand flies may have fed on the same reservoir. Additional sequence analysis will be necessary to determine the identity of the two *L. major/tropica*-like parasites and whether they represent a new species of *Leishmania* or are just a more divergent form of either *L. major* or *L. tropica.*

Saurian Leishmaniasis. One of our more surprising findings was that *L. tarentolae* accounted for >90% of the *Leishmania*-positive samples identified from sand flies at TAB, with *L. tarentolae*–infected sand flies collected at all sites throughout April to November. Although *L. tarentolae* has been widely used as a model system for biochemistry and cell biology studies (294 citations in PubMed), very little information is available on the natural biology of this blood-inhabiting protozoan. Additionally, confusion has surrounded the taxonomy of saurian *Leishmania* in general (Ovezmukhammedov and Saf'ianova 1989) and *L. tarentolae* specifically (Simpson and Holz 1988, Wallbanks et al. 1985). Overall, very little is known about the transmission of saurian *Leishmania* (Bates 2007). Known reservoirs of *L. tarentolae* are various species of geckos, to include *Tarentola mauritanica* L., *T. annularis* (Geoffroy Saint-Hilaire), and *Cyrtodactylus kotschyi* (Steindachner) (Wallbanks et al. 1985, Elwasila 1988), whereas *Sergentomyia* spp. sand flies have been implicated as vectors of this protozoan (Maroli et al. 1988). Abul-Hab and Ahmed (1984) reported that 8 of the 14 species of phlebotomine sand flies in Iraq belong to the genus *Sergentomyia*, with some species being the predominant phlebotomine sand fly in a given area and at certain times of the year. Although no effort was made to collect any reptiles at TAB, several species of small lizards were abundant and presumably served as reservoirs of *L. tarentolae.* Likewise, although we did not speciate any sand flies other than *Phlebotomus* spp., >45% of all sand flies collected at TAB were *Sergentomyia* spp. (Coleman et al. 2007).

Few studies have reported detection of *L. tarentolae* in sand flies. In one study in Italy, Maroli et al. (1988) detected *L. tarentolae* exclusively in *Sergentomyia minuta* Rondani. Sukkar (1985) and Sukkar et al. (1985) reported finding flagellates in the anterior midguts of 14 *S. baghdadis* (Adler and Theodor) and determined that flagellates in three of the four sand flies examined were *Leishmania* spp. Isoenzyme electrophoresis characterization of two of the *Leishmania* isolates indicated that they were unlike *L. infantum*, *L. donovani*, *L. tropica*, *L. major*, or *L. aethiopica* Bray, Ashford, and Bray; however, it was not determined whether they were *L. tarentolae.*

Because we did not separate *Phlebotomus* spp. from *Sergentomyia* spp. before testing, it was not possible to determine whether *L. tarentolae* was restricted to *Sergentomyia* spp. sand flies. However, because >45% of all phlebotomine sand flies that we identified at TAB were *Sergentomyia* spp. (Coleman et al. 2007), it is likely that most of the *L. tarentolae*--positive sand fly pools contained *Sergentomyia* spp. sand flies. To further assess whether *L. tarentolae* was restricted to *Sergentomyia* spp. sand flies, in 2005, we began separating *Sergentomyia* spp. from *Phlebotomus* spp. before testing. Of 12 pools of *Phlebotomus* spp. sand flies that tested positive for *Leishmania* DNA, *L. tarentolae* DNA was detected in 11 (92%) pools and *L. donovani*-complex parasites in 1 (8%) pool, whereas *L. tarentolae* was detected in all 13 (100%) pools of *Sergentomyia* sand flies that had tested positive for *Leishmania* DNA by using the real-time PCR assay. These data indicate that *Phlebotomus* spp. sand flies had acquired *L. tarentolae* DNA; however, the data does not allow us to determine whether viable parasites were present or whether the sand flies were capable of transmitting these parasites. Although we did not determine the source of blood contained in any of the field-collected *Phlebotomus* spp. sand flies, the data suggest that some of these flies had fed on an animal infected with *L. tarentolae*, presumably a lizard.

Comparison of Real-time PCR and Sequencing. In the absence of a true reference, it is difficult to determine the validity of a test result. The *Leishmania*-generic real-time PCR assay developed by Wortmann et al. (2001) is highly sensitive and specific for cultured *Leishmania* parasites, including *L. major*, *L. tropica*, and *L. donovani*-complex parasites, with a limit of detection of <10 parasites. Although comprehensive testing of this assay using laboratory-infected sand flies at the WRAIR had previously showed that this assay is both sensitive and specific for *L. major*, before this study we had little data on the performance of this assay for the detection of other species of *Leishmania* in sand flies. The data presented in Table 6 suggest that a Ct value that is >38 but ≤40 should be considered indeterminate, because only 3% of the samples in this range were considered positive after retesting. Assay repeatability increased markedly when Ct values were <38, with 91% of samples with an initial Ct value

between 36 and 37.99 considered positive after retesting and 100% considered positive when the initial Ct value was <36 (Table 6).

We had initially hoped that our sequencing results could be used to confirm the real-time PCR assay results; however, sequencing only detected *Leishmania* GPI in 54% (284/519) of the samples that were considered positive or indeterminate by PCR (Table 6). There was a clear relationship between the Ct value and the proportion of samples in which *Leishmania* GPI was detected, with the proportion of samples with *Leishmania* GPI increasing as the Ct value decreased. For example, *Leishmania* GPI was only detected in 22% of samples with a real-time PCR Ct value between 34 and 35.99, whereas *Leishmania* GPI was detected in 99% of samples with a Ct value of <26 (Table 6). Because the sequencing assays relied on nested PCR to generate enough DNA to work with, the nested PCR was probably not as sensitive as the real-time PCR assay for detecting the low quantity of parasite DNA present in the samples. Although we believe that the samples that were positive for *Leishmania* by real-time PCR but negative by sequencing should be considered true positives as long as the real-time PCR results are repeatable, we also believe that it is critical that a confirmatory *Leishmania*-generic PCR assay be developed. An ideal confirmatory assay would be as sensitive as or more sensitive than the original assay; however, would target a different *Leishmania* gene. Additionally, results from this study highlight the need for a variety of species-specific assays, as well as the need for an assay that could differentiate medically important *Leishmania* (e.g., parasites that cause visceral leishmaniasis, *L. major* and *L. tropica*) from nonmedically important *Leishmania* (e.g., *L. tarentolae*).

Summary. In this paper, we highlighted results of our *Leishmania* surveillance program at TAB, Iraq. Although our recent data identifying *L. tarentolae* as the predominate species of *Leishmania* in sand flies at TAB has tempered our concerns about the high infection rates initially reported in sand flies (Coleman et al. 2006), parasites that cause visceral leishmaniasis have emerged as a potential threat to military personnel deployed to TAB. Despite the low number of cases of visceral leishmaniasis reported in U.S. military personnel deployed to Iraq, we suggest that a significant number of soldiers, airmen, and marines stationed at TAB may have been exposed to parasites that cause visceral leishmaniasis. We believe that an FDA-approved method of assessing exposure of military service members to this parasite is urgently needed.

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References Cited

Abul-Hab, J., and S. A. Ahmed. 1984. Revision of the family Phlebotomidae (Diptera) in Iraq. J. Biol. Sci. Res. (Baghdad) 7: 1–64.

Adini, I., R. L. Jacobson, M. Kasap, Y. Schlein, and C. L. Jaffe. 1998. Species-specific detection of *Leishmania* in sand flies using an enzyme-linked immunosorbent assay. Trans. R. Soc. Trop. Med. Hyg. 92: 35–37.

Aransay, A. M., E. Scoulica, and Y. Tselentis. 2000. Detection and identification of *Leishmania* DNA within naturally infected sand flies by seminested PCR on minicircle kinetoplastic DNA. Appl. Environ. Microbiol. 66: 1933–1938.

Azizi, K., Y. Rassi, E. Javadian, M. H. Motazedian, S. Rafizadeh, M. R. Yaghoobi Ershadi, and M. Mohebali. 2006. *Phlebotomus* (*Paraphlebotomus*) *alexandri:* a probable vector of *Leishmania infantum* in Iran. Ann. Trop. Med. Parasitol. 100: 63–68.

Bates, P. A. 2007. Transmission of *Leishmania* metacyclic promastigotes by phlebotomine sand flies. Int. J. Parasitol. 37: 1097–1106.

Beier, J. C. 2002. Vector incrimination and entomological inoculation rates. Methods Mol. Med. 72: 3–11.

Bogdan, C., and M. Röllinghoff. 1998. The immune response to *Leishmania:* mechanisms of parasite control and evasion. Int. J. Parasitol. 28: 121–134.

Burkett, D. A., R. Knight, J. A. Dennett, V. Sherwood, E. Rowton, and R. E. Coleman. 2007. Impact of phlebotomine sand flies on U.S. military operations at Tallil Air Base, Iraq: 3. Evaluation of surveillance devices for the collection of adult sand flies. J. Med. Entomol. 44: 381–384.

Cardo, L. J. 2006. Leishmania: risk to the blood supply. Transfusion 46: 1641–1645.

Centers for Disease Control and Prevention. 2003. Cutaneous leishmaniasis in U.S. military personnel–Southwest/Central Asia, 2002–2003. Morb. Mortal. Wkly. Rep. 52: 1009–1012.

Centers for Disease Control and Prevention. 2004. Update: cutaneous leishmaniasis in U.S. military personnel—Southwest/Central Asia, 2002–2004. Morb. Mortal. Wkly. Rep. 53: 264–265.

Coleman, R. E., D. A. Burkett, V. Sherwood, J. Caci, L. Hochberg, and P. A. Weina. 2004. Prevention and control of leishmaniasis during Operation Iraqi Freedom. Wing Beats Fla Mosq. Control Assoc. 15: 10–16.

Coleman, R. E., D. A. Burkett, J. Caci, V. Sherwood, B. Jennings, L. Hochberg, P. A. Weina, and J. Putnam. 2005. Assessment of the leishmaniasis threat at Tallil Air Base, Iraq and development of a prevention and control program. Archives De L'Institut Pasteur de Tunis: Proc. 5th Int. Symp. Phlebotmine Sand Flies 82: 65.

Coleman, R. E., D. A. Burkett, J. L. Putnam, V. Sherwood, J. B. Caci, B. T. Jennings, L. P. Hochberg, S. L. Spradling, E. D. Rowton, K. Blount, et al. 2006. Impact of phlebotomine sand flies on U.S. military operations at Tallil Air Base, Iraq. 1. Introduction, military situation, and development of a "Leishmaniasis Control Program." J. Med. Entomol. 43: 647–662.

Coleman, R. E., D. A. Burkett, V. Sherwood, J. Caci, S. Spradling, B. T. Jennings, E. Rowton, W. Gilmore, K. Blount, C. E. White, et al. 2007. Impact of phlebotomine sand flies on U.S. Military operations at Tallil Air Base,

Iraq. 2. Temporal and geographic distribution of sand flies. J. Med. Entomol. 44: 29–41.

Colomba, C., L. Saporito, V. F. Polara, T. Barone, A. Corrao, and L. Titone. 2005. Serological screening for *Leishmania infantum* in asymptomatic blood donors living in an endemic area (Sicily, Italy). Transfus. Apher. Sci. 33: 311–314.

Dedet, J. P., and F. Pratlong. 2000. *Leishmania,* Trypanosoma and monoxenous trypanosomatids as emerging opportunistic agents. J. Eukaryot. Microbiol. 47: 37–39.

Dey, A., and S. Singh. 2006. Transfusion transmitted leishmaniasis: a case report and review of literature. Indian J. Med. Microbiol. 24: 165–170.

Eltoum, I. A., E. E. Zijlstra, M. S. Ali, H. W. Ghalib, M. M. Satti, B. Eltoum, and A. M. el-Hassan. 1992. Congenital kala-azar and leishmaniasis in the placenta. Am. J. Trop. Med. Hyg. 46: 57–62.

Elwasila, M. 1988. Leishmania tarentolae Wenyon, 1921 from the gecko *Tarentola annularis* in the Sudan. Parasitol. Res. 74: 591–592.

Fenech, F. F. 1997. Leishmaniasis in Malta and the Mediterranean basin. Ann. Trop. Med. Parasitol. 91: 747–753.

Fu, G., S. Melville, S. Brewster, J. Warner, and D. C. Barker. 1998. Analysis of the genomic organisation of a small chromosome of *Leishmania braziliensis* M2903 reveals two genes encoding GTP-binding proteins, one of which belongs to a new G-protein family and is an antigen. Gene 210: 325–333.

Gómez-Saladín, E., C. W. Doud, and M. Maroli. 2005. Short report: surveillance of *Leishmania* sp. among sand flies in Sicily (Italy) using a fluorogenic real-time polymerase chain reaction. Am. J. Trop. Med. Hyg. 72: 138–141.

Gramiccia, M., and L. Gradoni. 2005. The current status of zoonotic leishmaniases and approaches to disease control. Int. J. Parasitol. 35: 1169–1180.

Higgins, D. G., J. D. Thompson, and T. J. Gibson. 1996. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 266: 383–402.

Ivens, A. C., C. S. Peacock, E. A. Worthey, L. Murphy, G. Aggarwal, M. Berriman, E. Sisk, M. A. Rajandream, E. Adlem, R. Aert, et al. 2005. The genome of the kinetoplastid parasite, *Leishmania major.* Science 309: 436–442.

Jacobson, R. L. 2003. *Leishmania tropica* (Kinetoplastida: Trypanosomatidae)—a perplexing parasite. Folia Parasitol. (Praha) 50: 241–250.

Kinnamon, K. E., E. A. Steck, P. S. Loizeaux, L. D. Hendricks, V. B. Waits, W. L. Chapman, Jr., and W. L. Hanson. 1979. Leishmaniasis: military significance and new hope for treatment. Mil. Med. 144: 660–664.

Lawyer, P., and D. Young. 1991. Diapause and quiescence in *Lutzomyia diabolica* (Diptera:Psychodidae). Parasitologia. 33(Suppl): 353–360.

Lay, J. C. 2004. Leishmaniasis among U.S. Armed Forces, January 2003–November 2004. Medical Surveillance Mon. Report. U.S. Army Ctr. Health Promotion Prevent. Med. 10: 2–5.

Le Fichoux, Y., J. F. Quaranta, J. P. Aufeuvre, A. Lelievre, P. Marty, I. Suffia, D. Rousseau, and J. Kubar. 1999. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in southern France. J. Clin. Microbiol. 37: 1953–1957.

Magill, A. J., M. Grogl, R. A. Gasser, Jr., W. Sun, and C. N. Oster. 1993. Visceral infection caused by *Leishmania tropica* in veterans of Operation Desert Storm. New Engl. J. Med. 328: 1383–1387.

Maroli, M., M. Gramiccia, L. Gradoni, P. D. Ready, D. F. Smith, and C. Aquino. 1988. Natural infections of phlebotomine sandflies with Trypanosomatidae in central and south Italy. Trans. R. Soc. Trop. Med. Hyg. 82: 227–228.

Martin, S., J. Gambel, J. Jackson, N. Aronson, R. Gupta, E. Rowton, M. Perich, P. McEvoy, J. Berman, A. Magill, et al. 1998. Leishmaniasis in the United States military. Mil. Med. 163: 801–807.

Meinecke, C. K., J. Schottelius, L. Oskam, and B. Fleischer. 1999. Congenital transmission of visceral leishmaniasis (Kala Azar) from an asymptomatic mother to her child. Pediatrics 104: e65.

Michalsky, E. M., C. L. Fortes-Dias, P. F. Pimenta, N. F. Secundino, and E. S. Dias. 2002. Assessment of PCR in the detection of *Leishmania* spp in experimentally infected individual phlebotomine sandflies (Diptera: Psychodidae: Phlebotominae). Rev. Inst. Med. Trop. Sao Paulo 44: 255–259.

Molina, R., L. Gradoni, and J. Alvar. 2003. HIV and the transmission of *Leishmania.* Ann. Trop. Med. Parasitol. 97(Suppl 1): 29–45.

Morales, M. A., C. Chicharro, M. Ares, C. Cañavate, D. C. Barker, and J. Alvar. 2001. Molecular tracking of infections by *Leishmania infantum.* Trans. R. Soc. Trop. Med. Hyg. 95: 104–107.

Most, H. 1968. Leishmaniasis, pp. 1–28. *In* R. S. Anderson [ed.], Infectious diseases and general medicine. Office of the Surgeon General, U.S. Government Printing Office, Washington, DC.

Ostfeld, R. S., P. Roy, W. Haumaier, L. Canter, F. Keesing, and E. D. Rowton. 2004. Sand fly (*Lutzomyia vexator*) (Diptera: Psychodidae) populations in upstate New York: abundance, microhabitat, and phenology. J. Med. Entomol. 41: 774–778.

Ovezmukhammedov, A., and V. M. Saf'ianova. 1989. Taxonomic problems of the *Leishmania* of reptiles. Parazitologiia 23: 334–343.

Paiva, B. R., N. F. Secundino, J. C. Nascimento, P. F. Pimenta, E. A. Galati, H. F. Junior, and R. S. Malafronte. 2006. Detection and identification of *Leishmania* species in field-captured phlebotomine sandflies based on miniexon gene PCR. Acta Trop. 99: 252–259.

Peacock, C. S., K. Seeger, D. Harris, L. Murphy, J. C. Ruiz, M. A. Quail, N. Peters, E. Adlem, A. Tivey, M. Aslett, et al. 2007. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. Nat. Genet. 39: 839–847.

Pehoushek, J. F., D. M. Quinn, and W. P. Crum. 2004. Cutaneous leishmaniasis in soldiers returning from deployment to Iraq. J. Am. Acad. Dermatol. 51: S197–S200.

Riera, C., and J. E. Valladares. 1996. Viable *Leishmania infantum* in urine and semen in experimentally infected dogs. Parasitol. Today 12: 412.

Riera, C., R. Fisa, M. Udina, M. Gállego, and M. Portus. 2004. Detection of *Leishmania infantum* cryptic infection in asymptomatic blood donors living in an endemic area (Eivissa, Balearic Islands, Spain) by different diagnostic methods. Trans. R. Soc. Trop. Med. Hyg. 98: 102–110.

Rossi, E., G. Bongiorno, E. Ciolli, T. Di Muccio, A. Scalone, M. Gramiccia, L. Gradoni, and M. Maroli. 2008. Seasonal phenology, host-blood feeding preferences and natural *Leishmania* infection of *Phlebotomus perniciosus* (Diptera, Psychodidae) in a high-endemic focus of canine leishmaniasis in Rome province, Italy. Acta Trop. 105: 158–165.

Rosypal, A. C., and D. S. Lindsay. 2005. Non-sand fly transmission of a North American isolate of *Leishmania infantum* in experimentally infected BALB/c mice. J. Parasitol. 91: 1113–1115.

Rosypal, A. C., G. C. Troy, A. M. Zajac, G. Frank, and D. S. Lindsay. 2005. Transplacental transmission of a North American isolate of *Leishmania infantum* in an experimentally infected beagle. J. Parasitol. 91: 970–972.

Rosypal, A. C., G. C. Troy, A. M. Zajac, R. B. Duncan, Jr., K. Waki, K. P. Chang, and D. S. Lindsay. 2003. Emergence of zoonotic canine leishmaniasis in the United States: isolation and immunohistochemical detection of *Leishmania infantum* from foxhounds from Virginia. J. Eukaryot. Microbiol. 50(Suppl): 691–693.

Ryan, J., K. Davé, E. Emmerich, B. Fernández, M. Turell, J. Johnson, K. Gottfried, K. Burkhalter, A. Kerst, A. Hunt, et al. 2003. Wicking assays for the rapid detection of West Nile and St. Louis encephalitis viral antigens in mosquitoes (Diptera: Culicidae). J. Med. Entomol. 40: 95–99.

Ryan, J. R., K. Dave, E. Emmerich, L. Garcia, L. Yi, R. E. Coleman, J. Sattabongkot, R. F. Dunton, A. S. Chan, and R. A. Wirtz. 2001. Dipsticks for rapid detection of *Plasmodium* in vectoring anopheles mosquitoes. Med. Vet. Entomol. 15: 225–230.

Sakru, N., M. Korkmaz, Y. Ozbel, H. Ertabaklar, M. Sengul, and S. O. Toz. 2007. Investigation of asymptomatic visceral leishmaniasis cases using western blot in an endemic area in Turkey. New Microbiol. 30: 13–18.

Simpson, L., and G. Holz, Jr. 1988. The status of *Leishmania tarentolae*/*Trypanosoma platydactyli.* Parasitol. Today 4: 115–118.

Sukkar, F. 1974. Study on sandflies as vectors of kala azar in Iraq. Bull. Endemic Dis. (Baghdad) 15: 85–104.

Sukkar, F. 1985. Epidemiology and control of visceral leishmaniasis in Iraq. Bull. Endemic Dis. (Baghdad) 26: 13–26.

Sukkar, F., F. Yawanis, N. A. Al-Doori, and S. K. Al-Mahdawi. 1985. The possible vector of infantile leishmaniasis in Iraq. Bull. Endemic Dis. (Baghdad) 26: 27–36.

Svobodova, M., and J. Votýpka. 2003. Experimental transmission of *Leishmania tropica* to hamsters and mice by the bite of *Phlebotomus sergenti.* Microbes. Infect. 5: 471–474.

Svobodová, M, J. Votýpka, L. Nicolas, and P. Volf. 2003. *Leishmania tropica* in the black rat (*Rattus rattus*): persistence and transmission from asymptomatic host to sand fly vector *Phlebotomus sergenti.* Microbes Infect. 5: 361–364.

Wallbanks, K. R., R. Maazoun, E. U. Canning, and J. A. Rioux. 1985. The identity of *Leishmania tarentolae* Wenyon 1921. Parasitology 90: 67–78.

World Health Organization. 2003. Communicable disease profile—Iraq. Communicable Disease Working Group on Emergencies, HQ Division of Communicable Disease Control, EMRO, WHO Office, Baghdad, Iraq.

Wortmann, G., C. Sweeney, H.-S. Houng, N. Aronson, J. Stiteler, J. Jackson, and C. Ockenhouse. 2001. Rapid diagnosis of leishmaniasis by fluorogenic polymerase chain reaction. Am. J. Trop. Med. Hyg. 65: 583–587.

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